Master's Thesis

Revealing the Role of Histone Tails in Controlling Nucleosome Assembly and Chromatin Compaction

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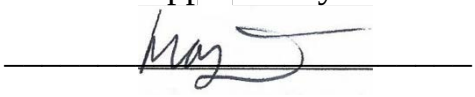

Advisor
Hajin Kim

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Hongsoo Lee

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Abstract

Histone proteins are essential to the construction of chromatin fiber and controlling its conformational state for the regulation of gene expression. DNA wrapping over histone octamer forms a nucleosome as the basic unit of chromatin fiber and understanding how the nucleosome wrapping/positioning and the compaction of nucleosome arrays are controlled is the key to understanding epigenetic gene regulation. Each histone protein subunit has unstructured tail region, which is known as multiplexed target of epigenetic modification. But we still lack knowledge about the role of the tail regions and the effect of each epigenetic modification on the chromatin structure and dynamics. We developed magnetic tweezers combined with single molecule fluorescence imaging to measure the mechanical properties and dynamics of nucleosomes and chromatin fibers. By using histone proteins with deleted tail regions or with epigenetic modification at specific residues, we try to figure out their role in controlling chromatin structure and dynamics. We present preliminary results from the measurement of the chromatin compaction and nucleosome unwrapping dynamics.

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Figure 17. Disassembly length of wildtype nucleosome and every modified nucleosome.

Nomenclature

4D: Histone H4 tail deletion

ABD: Histone H2A/H2B tail deletion

AB4D: Histone H2A/H2B/H4 tail deletion

MNase: Micrococcal Nuclease

NRL: Nucleosome Repeat Length

NAP: Nucleosome Assembly Protein

WLC: Worm Like Chain

WT: Wild Type

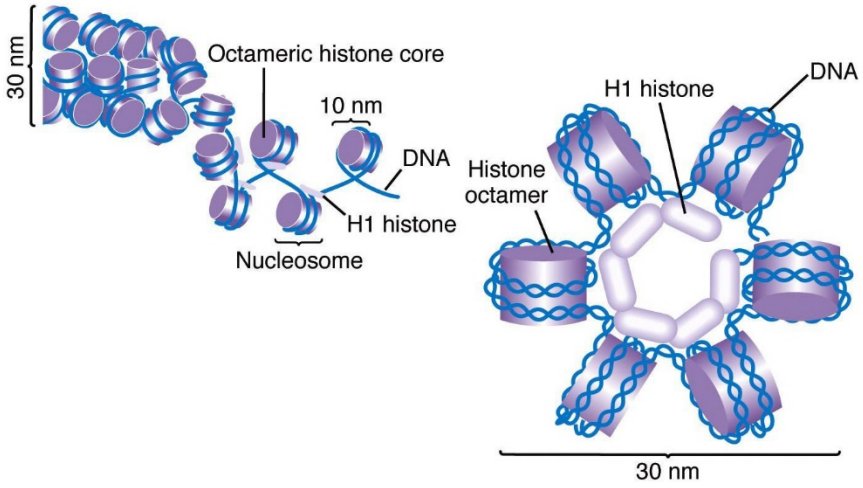
1. Introduction

DNA wrapping over histone octamer forms a nucleosome which is the basic unit of chromatin fiber and the epigenetic modifications existing on the level of DNA, histones, and nucleosomes is known to control gene expression. Epigenetic studies mainly focus on the effect of DNA methylation, histone modifications, histone variants, and nucleosome conformation. In addition, modified histone tails, which is known as multiplexed target of epigenetic modification considered as key pathway for chromatin compaction, thus influencing gene expression. But we still lack the knowledge about the role of each epigenetic modification on histone tail to the chromatin structure and dynamics.

In this experiment, we try to figure out the role of histone tails by measuring chromatin structure and dynamics through home-built magnetic tweezers.

1.1. DNA and Histone Protein

Histone protein and DNA are the basic repeating units of eukaryotic chromatin, which forms secondary structure called nucleosome. It makes DNA able to highly be condensed and forms chromosomes. Because histone protein has positive charges, it allows negatively charged DNA associate to histone protein and make nucleosome. Each nucleosome is composed of eight histone proteins which is wrapping DNA and are called as histone octamer. Each histone octamer is made of two of the histone subunit called H2A, H2B, H3, and H4. The successive nucleosomes then form a 30nm spiral which looks like solenoid, where additional histone proteins called as H1 are associated to nucleosome to form solenoid structure[1,7].

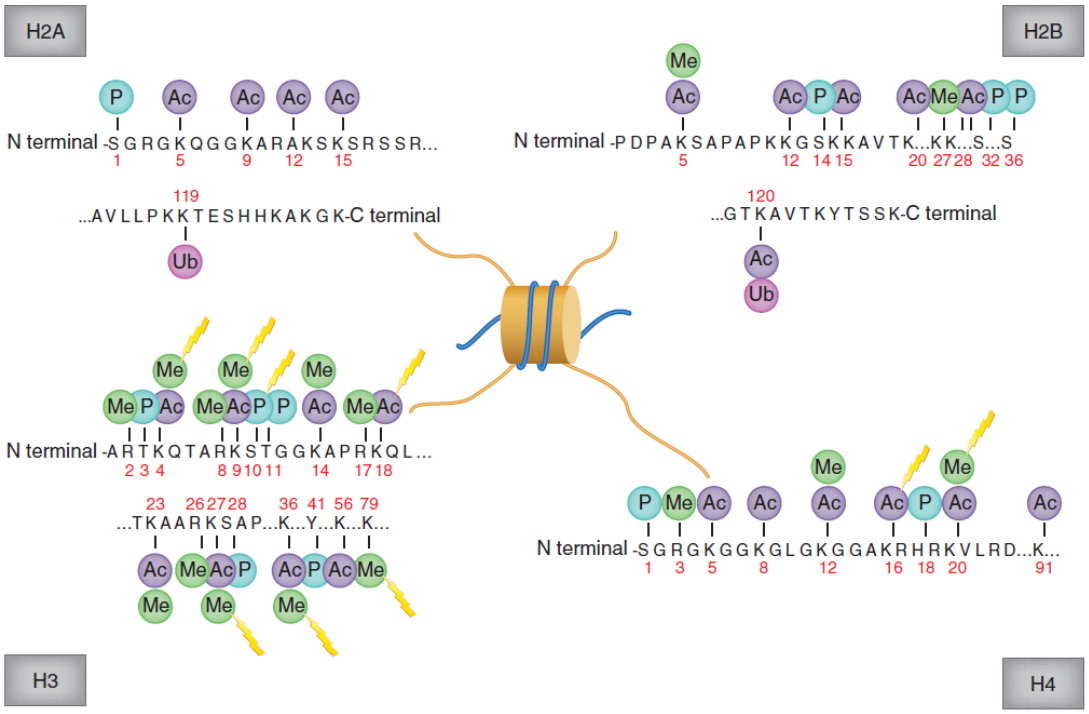


https://www.mun.ca/biology/scarr/Histone_Protein_Structure.html

Figure 1. Stepwise view of histone/DNA to nucleosome structure

1.2 Histone Tail and its Epigenetic Modification Site

Each histone protein subunit has unstructured tail region on its N-terminal, which plays a key role in gene transcription and expression and is known as multiplexed target of epigenetic modification. Many studies have revealed that several combinations of epigenetic combinations in a specific chromosome region can lead gene to a more ‘closed’ or ‘open’ state of chromatin structure and, therefore, to the activated or repressed state of gene expression[2].



Manual Rodíguez-Paredes et al., Nature Medicine 17, 330-339 (2011)

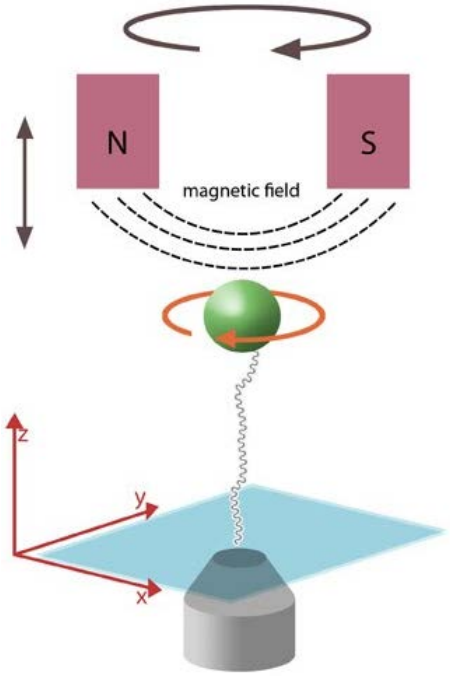
Figure 2. Epigenetic modification sites on histone Tails

Each lysine residues can go through methylation or acetylation which result in gene expression or repression. For example, Trimethylation of lysines on H3 tail (K4, K36, K79 respectively) and monomethylation on H4 tail and H2 tail (H4K20, H2BK5) is known to activate genes, whereas trimethylation of H3K9 and H3K27 is known to repress gene expression.

All these epigenetic processes work together to maintain the global and local condensed or decondensed chromosome states that finally determine gene expression state. The continuous interaction of these works to maintain the epigenetic status of eukaryotic genome works for deciding its cell types and developmental stages and, sometimes to cancer and other diseases.

1.3 Magnetic Tweezers for Nucleosome Dynamics

To figure out the role of histone tails on chromatin structure and its stability, we used single-molecule magnetic tweezers to examine whether variant histone tails will give any structural difference on nucleosome[3]. Magnetic tweezers are quite suited to show this structural difference, because this tool permit us to track the individual magnetic bead position, and by tracking the magnetic bead's position, we can get the nucleosome wrapping/unwrapping events by showing discrete position change of magnetic bead. It also allows for showing structure difference between wildtype histone and tail-modified histone in nucleosome compaction by measuring force which acts between nucleosomes.



http://mbinfo.mbi.nus.edu.sg/figure/1415958213461/

Figure 3. Diagrammatic representation of a basic vertical magnetic tweezers

2. Materials and Method

2.1 16x197 NRL sequence preparation

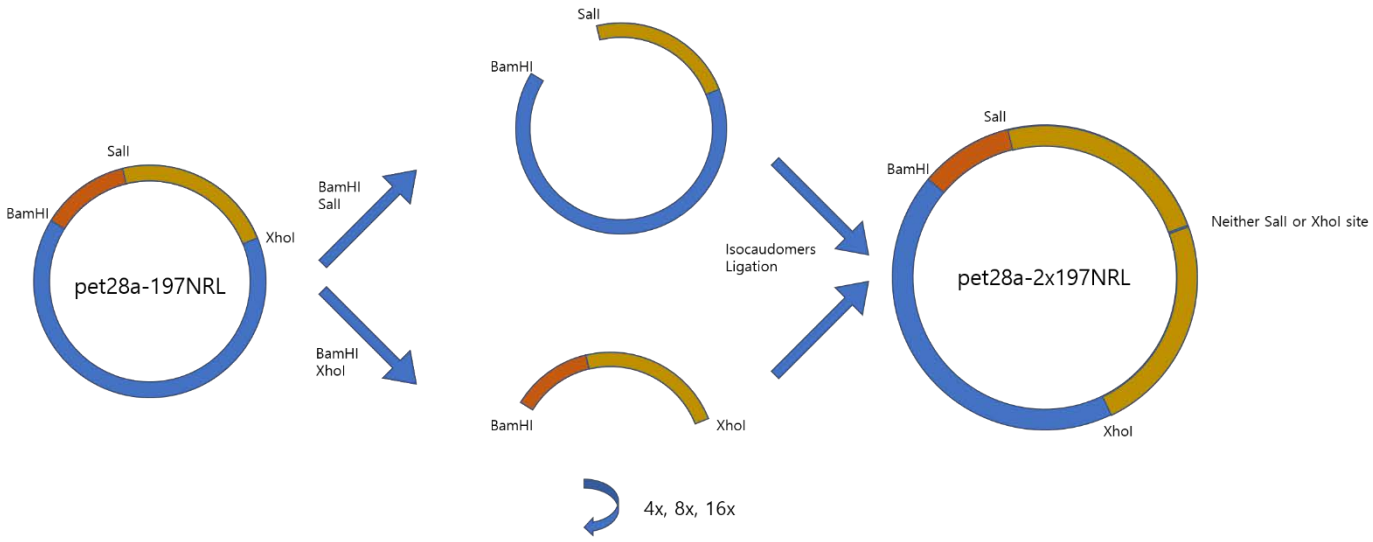


Figure 4. Schematic process of 16x 197NRL sequence production

In plasmid backbone pet28a (from Addgene), 147base Widom sequence and 50base linker sequence (197NRL) is inserted between BamHI and XhoI restriction enzyme site. And near BamHI site, we inserted SalI site which is isocaudomer to XhoI. After making pet28a with 1x 197NRL plasmid, for making vector which has 1x 197NRL, pet28a-197NRL plasmid was treated with BamHI and SalI. And for making 1x 197NRL insert, pet28a-197NRL plasmid was treated with BamHI and XhoI.

Since SalI and XhoI is isocaudomer, SalI site of backbone and XhoI site of insert can be ligated and make sequence which is not recognizable to neither SalI or XhoI. And final product has 2x197NRL sequence in pet28a. Through this step, 197NRL sequence has amplified to 16x in pet28a backbone.

2.2 Handle Sequence preparation

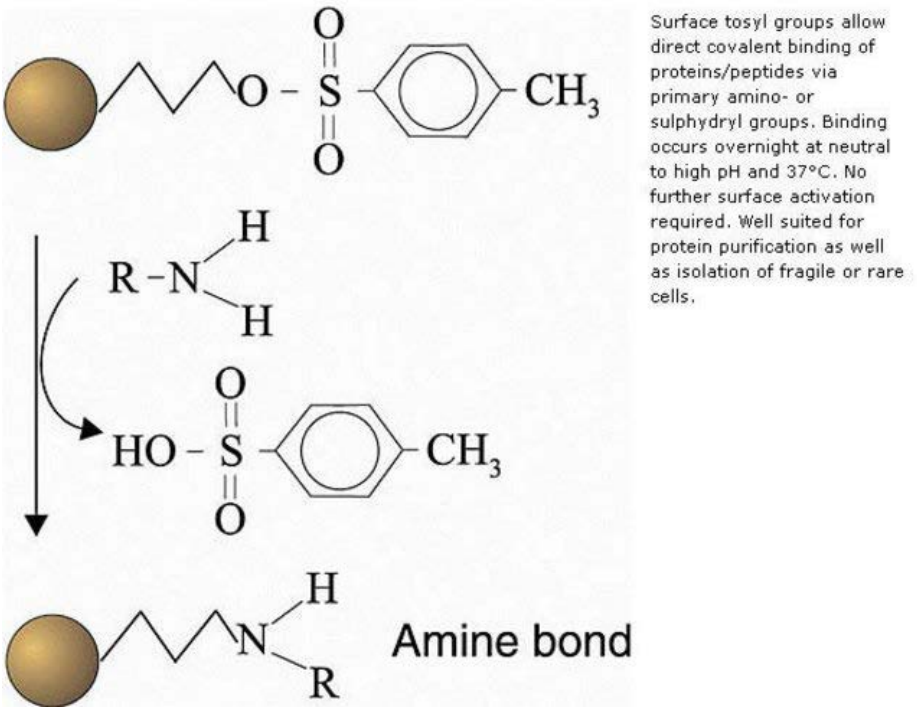
For preventing magnetic bead freely rotate, 16x197 NRL sequence is ligated to handle sequence which has length of 500 basepair and multiple modified nucleotide. Each handle was designed same to pet28a sequence near BamHI site and XhoI site. Each sequence was constructed by PCR from pet28a backbone with Phusion PCR MM (NEB), biotin-dUTP (Jena Bioscience), and dig-dUTP (Jena Bioscience). BamHI site of pet28a is amplified for biotin handle and XhoI site for dig handle. For PCR, the following reagents was used with this ratio:

Phusion MM	25ul (supplemented with 0.2mM of each dNTPs)
Forward Primer	2.5ul
Reverse Primer	2.5ul
Biotin-dUTP (1mM)	1.5ul (only for biotin handle)
Dig-dUTP (1mM)	1.5ul (only for dig handle)
Template DNA	1pg
DEPC	up to 50ul

With the ratio between modified dUTP and normal dTTP of 1:3, we can expect final handle product may have approximately thirty biotin or dig sites which make handle can bind to multiple site of neutravidin on slide or anti-dig site on M280 magnetic bead.

After constructing each handle sequence, 16x 197NRL sequence (approx. 3200bp) and each handle sequence (500bp) was ligated in 16C overnight. The ratio between insert and handle sequence was 10:1 for preventing self-ligation of 16x 197NRL sequence. The ligated product was run on gel and the band on 4200bp (H1 + 16x + H2) was cut and gel purified[4, 6, 8].

2.3 Magnetic Bead Coating



https://www.thermofisher.com/order/catalog/product/65501

Figure 5. Direct covalent binding of proteins and peptides to Dynabeads Myone Tosylactivated

Because tosylactivated M-280 is coated with sulphydryl groups which can react to amine group in protein. It allows the bead directly to bind to protein with covalent bond. The following method was used for M-280 coating:

10mg(335ul) of tosylactivated M-280 was washed twice with 0.1M sodium PBS ph7.4 and mixed with 100ug of anti-digoxigenin (Roche) in 150ul of 0.1M sodium PBS ph7.4. And 100ul of 3M ammonium sulphate was added. The mixture was incubated on a roller at 37°C for 12 ~ 18 hours.

The tube was placed on a magnet for 2 min and washed with PBS with 0.5%(w/v) BSA. Then incubated again on a roller at 37°C for 2 hours or longer. Finally, the mixture was washed with PBS with 0.1%(w/v) BSA twice and resuspended to 480ul of PBS with 0.1%(w/v) BSA with making final concentration of 20mg/mL[10].

2.4 Magnetic Tweezers

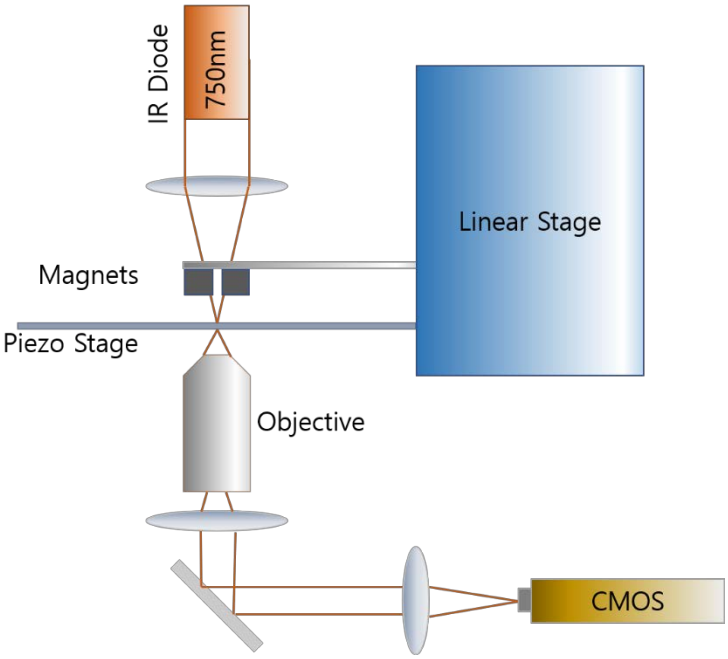


Figure 6. Schematic view of magnetic tweezers

The LED source (Thorlabs, 750nm) was used for making the diffraction pattern image. And CMOS camera (SP-5000-usb, JAI) was used for imaging the diffraction pattern of magnetic beads. For controlling force on magnetic beads, we used linear stage (PLS-85, PI Korea) which has neodymium magnets on edge of stage. Two magnets were fixed on the edge of stage with the gap of 1mm. The gap of 1mm between magnet is known to give magnetic force up to 70pN. For Z-position calibration, we used piezo stage (FT-2150, ASI) which make us enable to control the stage precisely and track the z-position of magnetic bead accurately.

2.5 Tweezers Sample Preparation

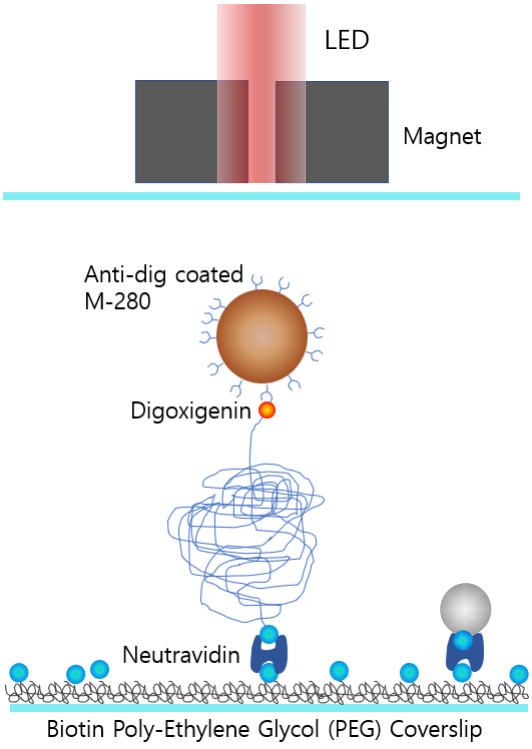


Figure 7. Schematic structural view of magnetic tweezers sample

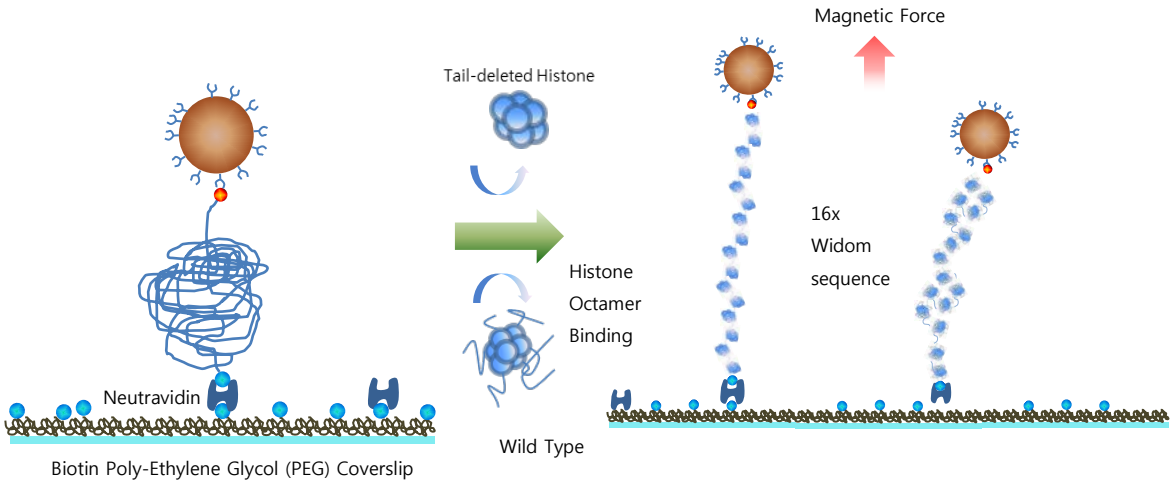


Figure 8. Schematic workflow of real-time observation of nucleosome disassembly

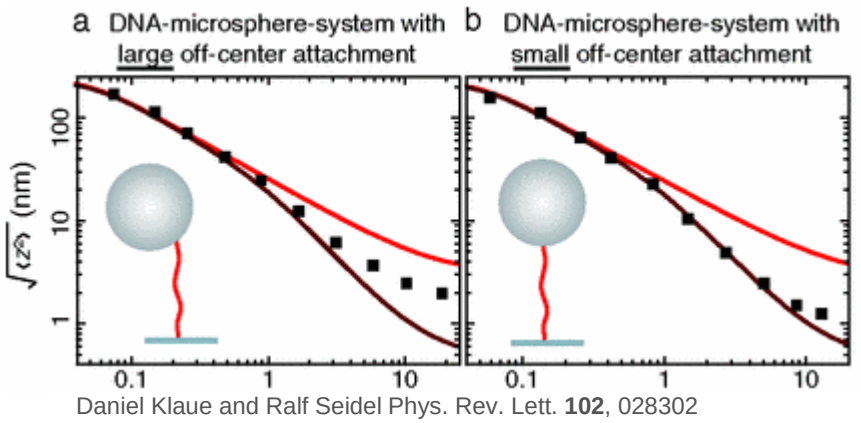
The coverslip is coated with PEG (Poly-Ethylene Glycol) and biotin-PEG with the ratio of 40:1 (PEG: biotin-PEG). After incubating neutravidin for 5min, pre-assembled magnetic bead and 16x 197NRL sequence was injected and incubated for 15min (The magnetic bead and NRL sequence was

pre-incubated for several hours with the ratio of 0.5:1 to prevent multiple binding of DNA). The channel was washed twice, then, reference bead was injected and incubated for 15min.

For nucleosome assembly, 6nM of histone octamer and 2nM of NAP1L1 was injected to channel and incubated for 15min. When the histone protein was injected, 3pN of magnetic force was given to magnetic bead to prevent bead from being bound to slide non-specifically.

After nucleosome reconstitution, with 0.1pN/s of force increment, magnetic bead position was observed in real-time. As the magnetic force increase, compaction of histone or disassembly of nucleosome will be shown as position change of magnetic bead[5].

2.6 Code for preventing to pick the off-center attachment of bead



Daniel Klaue and Ralf Seidel Phys. Rev. Lett. **102**, 028302

Figure 9. Schematic image showing Off-center attachment show bad worm like chain fitting. WLC curve (brown), and dotted line (measured).

In this article, they proved if the DNA is attached off center, the extension curve will not show exact dynamics of DNA and histone protein[9]. To solve this problem, we made this code:

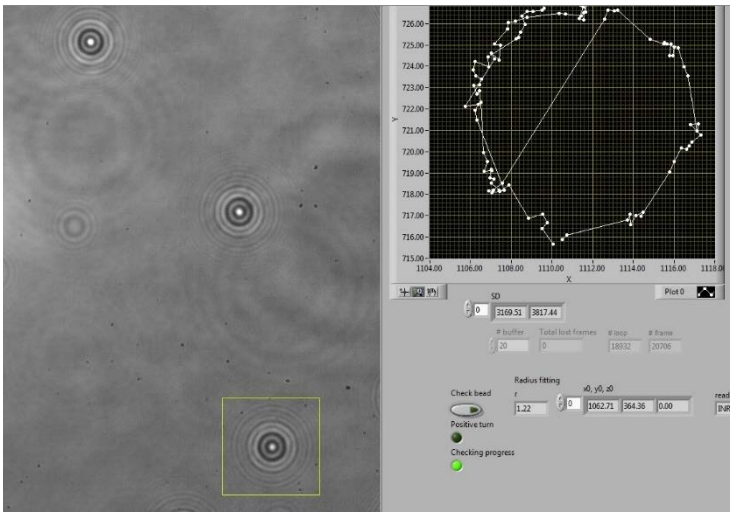


Figure 10. Front panel of bead-picking code

Before picking appropriate bead for tracking, beads were rotated clockwise and anti-clockwise once for measuring the diameter of rotation. The center position of magnetic bead was tracked, and the diameter of rotation was calculated by measuring pixel of camera. If the diameter exceeds 1um, a bead was excluded from observation because of off-center attachment which will interfere the real-time observation of magnetic bead.

3. Results

3.1 Construction of plasmid having 16x 197NRL sequence

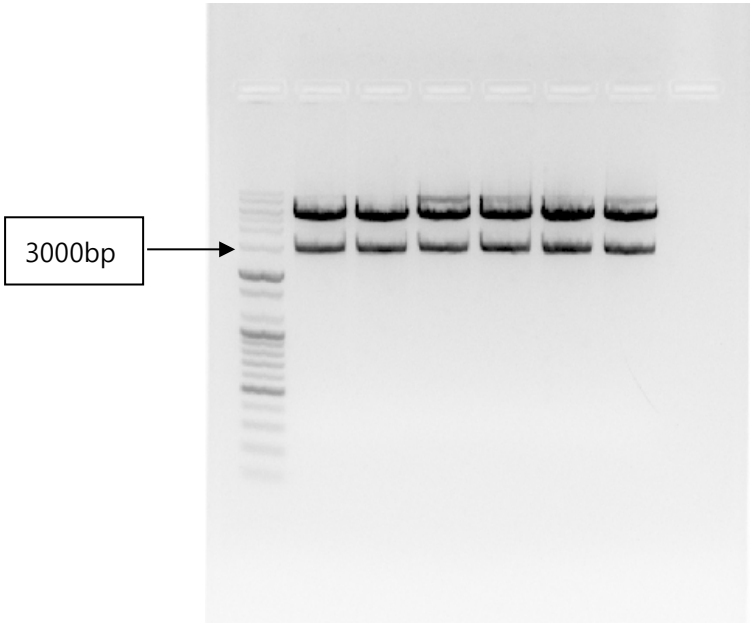


Figure 11. BamHI / XhoI Restriction results of PM50-16x 197NRL

Since a repeating sequence is not well amplified with polymerase chain reaction(PCR), to confirm the construction of plasmid which has 16x 197NRL sequence, we used digestion of final plasmid with BamHI and XhoI. Through this digestion result of the pet28a-16x 197NRL, we can confirm that 16x197NRL sequence has successfully made into PM50 vector and stably expressed with STBL3 competent bacterial cells.

3.2 Histone Binding Confirmation through agarose gel

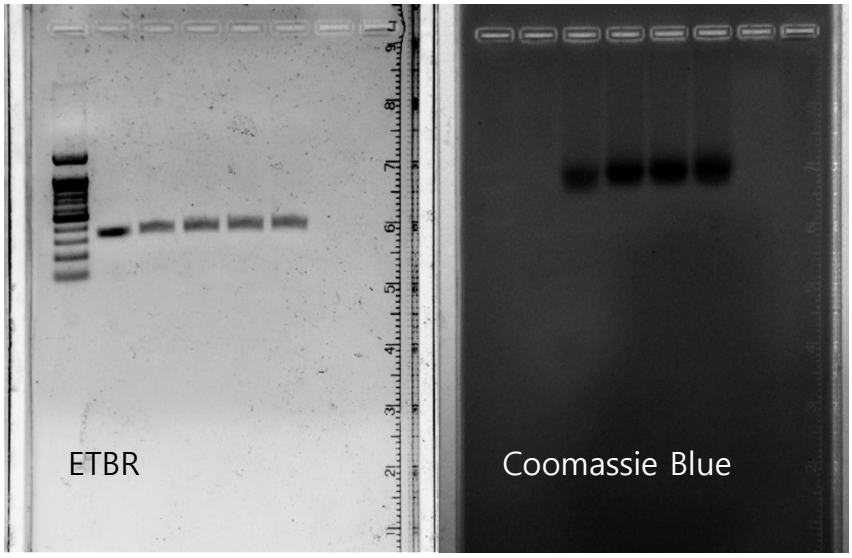


Figure 12. Confirmation of biding of histone protein to Widom sequence through agarose gel

To confirm the binding affinity of histone protein to Widom sequence, each histone subunit combination (From left; DNA Ladder, without histone, AB4D, ABD, 4D) was incubated with 2x197NRL sequence. ETBR stained gel show the ratio of histone bound DNA and unbound DNA (Left). And for visualize histone protein, agarose gel was fixed and stained with Coomassie blue (Right). Histone protein appeared around 1.2kb region which is bigger as 1kb than bare DNA.

Histone proteins are known as positively charged proteins to wrap the negatively charged DNA, so, we expected the protein will proceed to up and, if it is bound to DNA, it will go down. Since, we observed the Coomassie blue band go down, we concluded that histone protein is wrapping the DNA.

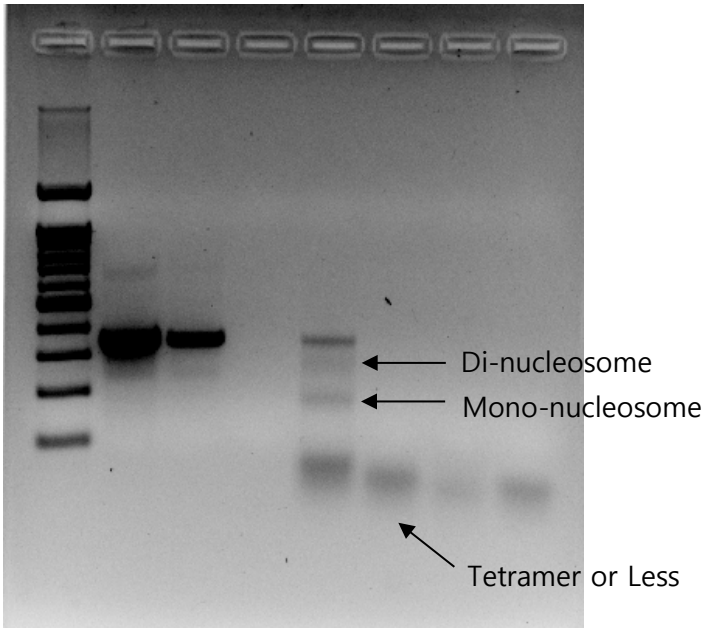


Figure 13. Agarose gel image of MNase treated nucleosome reconstitution. (1. Ladder; 2. DNA w/o Phenol/Chloroform extraction; 3. DNA after Phenol/Chloroform extraction; 4. Bare DNA after MNase treatment; 5. WT nucleosome after MNase treatment; 6. AB4D nucleosome after MNase treatment; 7. ABD nucleosome after MNase treatment; 8. 4D nucleosome after MNase treatment)

Because MNase give more direct information about nucleosome reconstitution, we reconfirmed nucleosome reconstitution with MNase treatment. Third Lane shows phenol/chloroform extraction has efficiency of 50% and fourth lane sample was incubated bare DNA (1ug) with Mnase and it shows MNase is functioning well for digestion. Reconstituted WT nucleosome after MNase treatment shows WT nucleosome is formed well by showing the intense band around 170bp region, whereas the other modified nucleosome after MNase treatment shows its band smaller than 100bp which means nucleosome is reconstituted with only tetramer and has low efficiency. It suggests us histone tail has some interaction with nucleosome assembly protein (NAP1L1) which helps histone protein form nucleosome.

3.3 Real-time observation of nucleosome disassembly events with magnetic tweezers

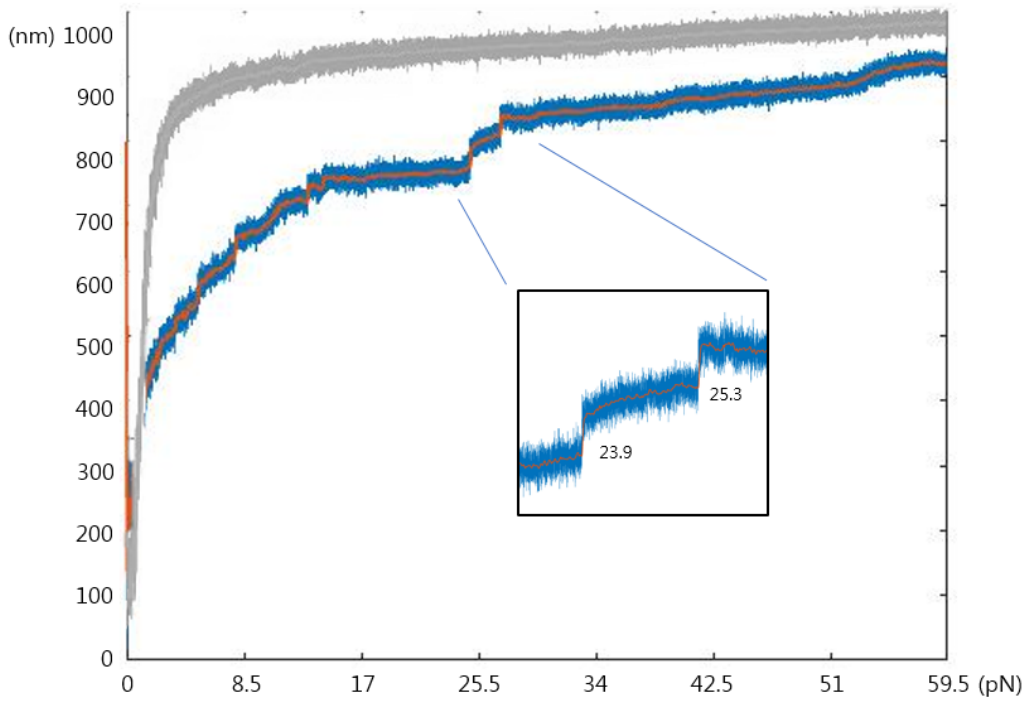


Figure 14. Real-time observation of wildtype nucleosome disassembly

To study the stability of wildtype nucleosomes dependent to force, we measure the disassembly event of WT histone. After assembling nucleosome on NRL sequence, we increased the magnetic force on the magnetic bead with a speed of 0.1pN/s, and the position of magnetic bead was tracked simultaneously in real-time. While this magnetic force change resulted in the WLC fitting bare DNA extension, in case of WT histone assembled DNA, additional discrete changes of bead position were observed because of the disassembly of individual nucleosome, which is the release of Widom sequence wrapping around the nucleosome. Through this real-time observation of nucleosome disassembly, we could observe wildtype histone protein has high efficiency for nucleosome reconstitution and, on average, wildtype nucleosome disassembly has stepsize of around 24nm which coincide to length of nucleosome inner-turn.

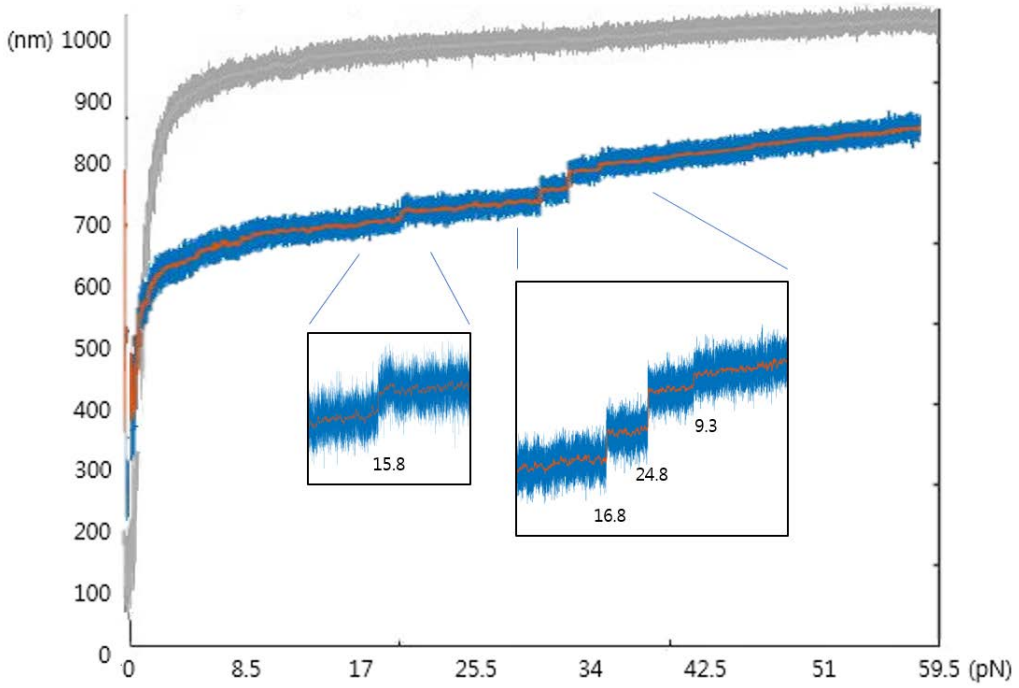


Figure 15. Real-time observation of H4-tail deleted nucleosome disassembly

To investigate the role of histone H4-tail on physical property of nucleosome, we also observe H4-tail deleted nucleosome disassembly in real-time. Similar to wildtype nucleosome tracking, H4-tail deleted nucleosome showed discrete changes of magnetic bead position which means the disassembly of histone proteins from DNA. Through tracking H4-tail deleted nucleosome, we could recognize that the average number of discrete change of magnetic bead position has lowered significantly compared to wildtype histone. Moreover, the stepsize of disassembly has shortened to 30~50% of stepsize from wildtype. It is suggesting that, when H4-tail deletion is given, the nucleosome reconstitution is not as complete as wildtype and it also lower the efficiency of nucleosome reconstitution.

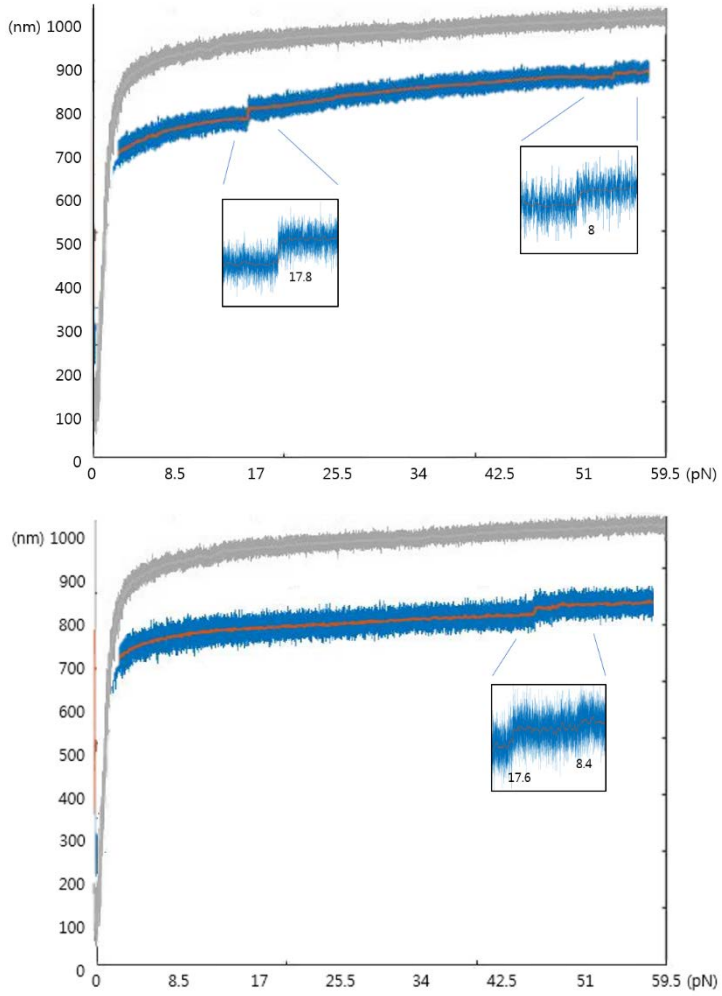
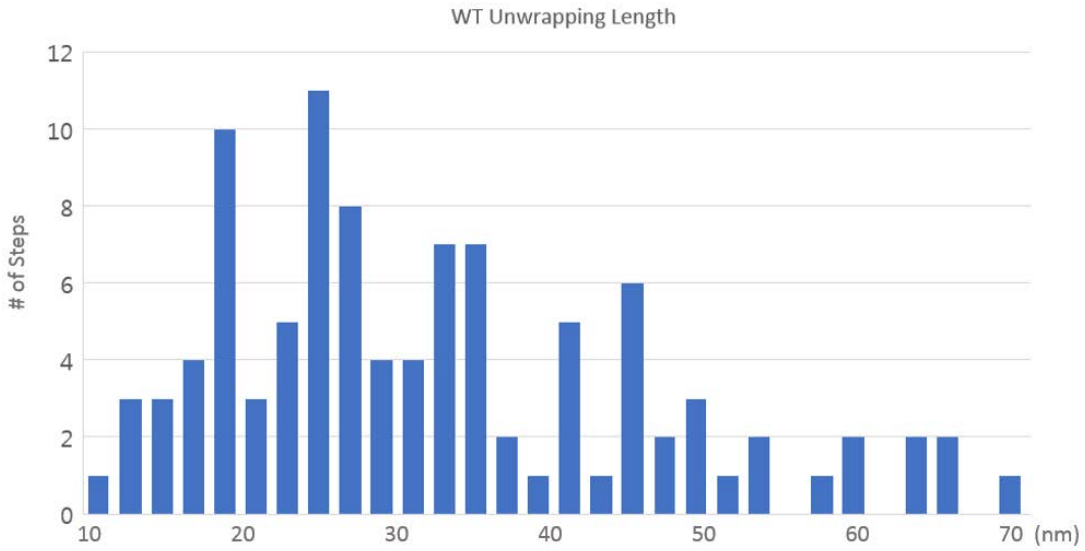
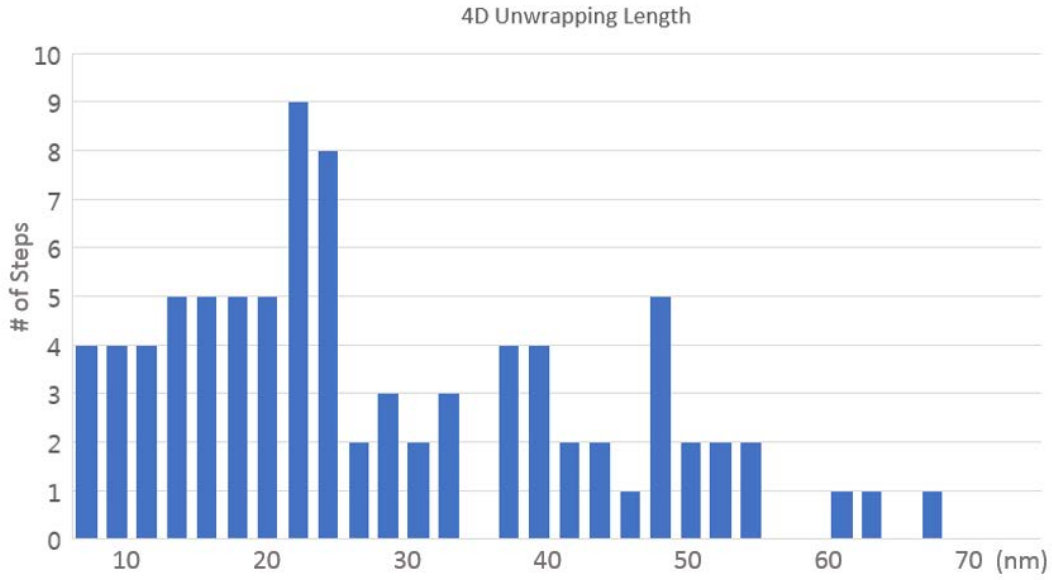


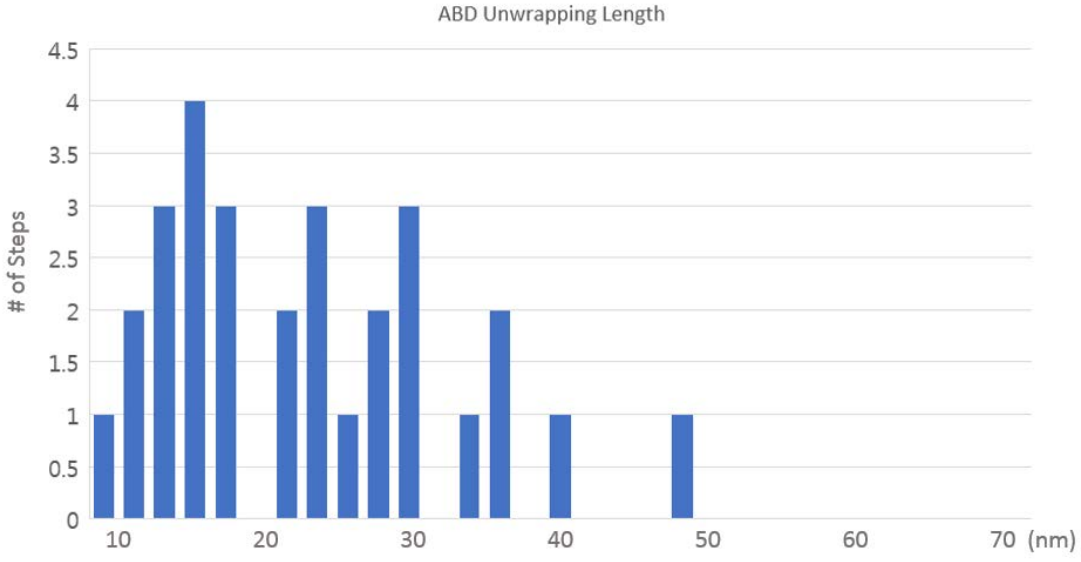
Figure 16. Real-time observation of disassembly event of H2A/H2B-tail deleted (Top) and H2A/H2B, H4-tail deleted (Bottom) nucleosome

Also, H2A/H2B-tail deletion was introduced to the 16x 197NRL tethered on slide. H2A/H2B-tail deleted nucleosome showed different dynamics compared to wildtype or H4-tail deleted nucleosome by its disassembly stepsize and the number of discrete changes of magnetic bead position. Firstly, the number of sudden jumps of magnetic bead position is fewer than that of wildtype and H4-tail deleted nucleosome. It suggests that the reconstitution efficiency even more reduced and H2A/H2B tails are related to NAP1 working efficiency. Also, the disassembly stepsize was lowered compared to wildtype which is similar to H4-tail deleted nucleosome compared to wildtype.

3.4 Stepsize of nucleosome disassembly







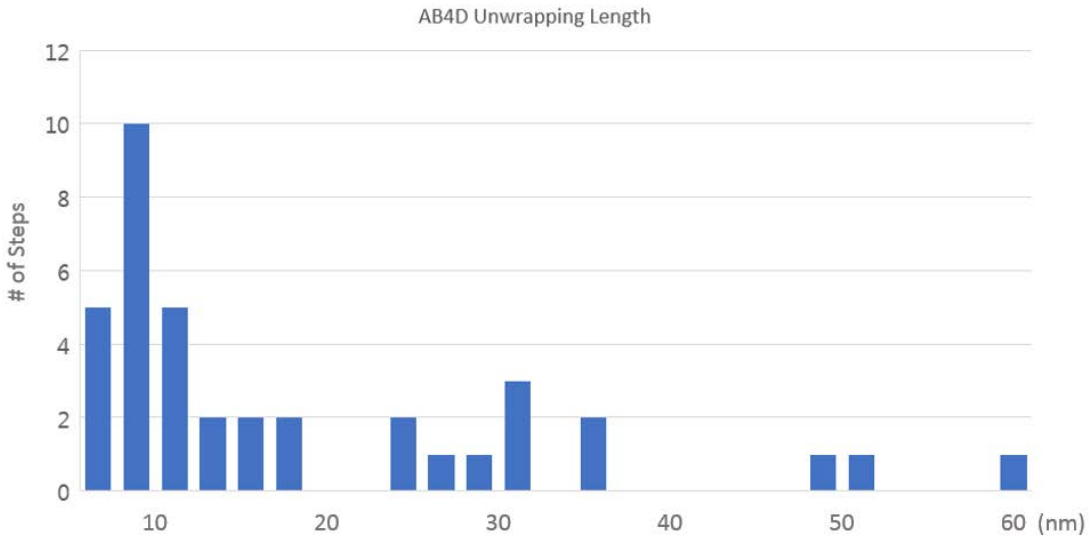


Figure 17. Disassembly length of wildtype nucleosome and every modified nucleosome.

To investigate the structural difference between wildtype and modified nucleosomes, we measured the disassembly step-length of every type of modifications on nucleosome from traces. Firstly, wildtype disassembly length histogram has most its population on 22~26nm which is known as inner-turn of normal nucleosome. And it also shows populations on 42~48nm and 60~66nm which represent di-nucleosome and tri-nucleosome disassembly length. Also, wildtype nucleosomes show a lot of discrete changes of magnetic bead position which means it has most efficient ability for nucleosome reconstitution.

When we measure the disassembly step of H4 tail deleted nucleosome, the number of population which has step size bigger 22nm has lowered, whereas, every other population was similar to wildtype's disassembly population. It suggests us that H4 tail has role on making higher structure of nucleosome such as nucleosome outer-turn or some part of nucleosome inner-turn.

Also, H2A/H2B-tail deleted nucleosome disassembly was measured by measuring the length of discrete changes of traces, H2A/H2B-tail deleted nucleosome shows more loosened structure by showing its disassembly population on 16~18nm mostly. And, from H2A/H2B-tail deletion, the number of discrete jumps from trace become very few. It suggests us H2A and H2B tails are related to stability of inner-turn of nucleosome by showing its stepsize and reconstitution efficiency has very lowered than WT and H4-tail deleted.

When we introduced H2A/H2B/H4-tail deletion to nucleosome, the disassembly stepsize has significantly lowered compared to wildtype, H4-deletion, and H2A/H2B-deletion. Most of its stepsize located lower than 10nm which suggest it almost lose its ability to make nucleosome inner-turn and outer-turn by itself. Also, number of changes in trace also become fewer than even that of H2A/H2B-tail deleted nucleosome.

4. Conclusion& Discussion

In this dissertation, we built the magnetic tweezers for tracking the dynamics of DNA and histone protein, and compared the stability of wildtype nucleosome and tail-deleted nucleosome, in order to test the hypothesis that histone tails may have roles on nucleosome stability and chromatin compaction.

Out home-built magnetic tweezers successfully worked for picking the beads which don't show off-center attachment and tracking 3D position of magnetic bead which shows sudden changes on magnetic bead position in real-time which can be considered as the disassembly of nucleosome.

Correspond to our hypothesis of changes on nucleosome stability, our MNase assay data and magnetic tweezers data show that histone tails have several roles on nucleosome stability, nucleosome reconstitution efficiency on Widom 601 sequence which may be related to nucleosome assembly protein (NAP1), which is supported by known H2A/H2B tail's interaction with NAP1.

Firstly, our MNase gel data showed the nucleosome reconstitution ability of each modified histones and wildtype histone is different. Wildtype histone protein showed that it works well by high intensity band around 170bp which corresponds to 1xWidom sequence, whereas, the other modified histone showed its band only on smaller than 100bp which may mean histone protein forms only tetramer or less.

And our magnetic tweezers data show that the histone tail has some roles on reconstituting and maintaining the inner or outer turn of nucleosome by showing the differences in stepsize of nucleosome disassembly. As we delete more histone tails, the stepsize of disassembly and number of disassembly was lowered which means binding affinity and wrapping length has lowered.

In conclusion, the single-molecule experiments shown that H2A/H2B tail and H4 tail have roles on maintaining the nucleosome structure and, moreover, it also intervenes to nucleosome reconstitution when we use NAP1 for reassembly of histone protein. These data provide insights into each histone-tail's role on nucleosome stability and basis for future works which will work for revealing specific epigenetic modifications on histone tails.

Possible further research as an extension to this work includes the following suggestions:

- Dialysis for reconstituting the nucleosomes with modified histones: because modified histone has low efficiency for reconstituting nucleosome with NAP1, it was hard to figure out and gather its physical property. By dialyzing for reconstituting, it will be easier to do other works using nucleosome.

- Effect of specific modifications in histone tail on nucleosome stability: this research can be repeated in same way with another epigenetic modification to reveal the role of that modification's role on nucleosome stability

- Effect of polyamine on nucleosome stability: as the ion concentration become high, it is known that nucleosome become unstable. But it is not clear yet whether polyamine has its role on nucleosome stability. If it induces nucleosome to be unstable, we can do further research to reveal the relation between polyamine and transcription efficiency.

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